Deuterium Exchange of *C*-Methyl Protons in 6,7-Dimethyl-8-D-ribityl-lumazine, and Studies of the Mechanism of Riboflavin Biosynthesis

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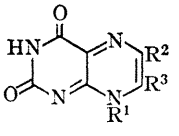
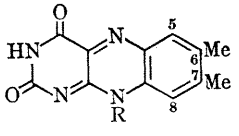
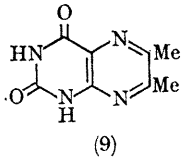
Summary The 7-methyl protons of 6,7-dimethyl-8-D-ribityl-lumazine and some analogues exchange with deuterium, and the deuteriated 8-hydroxyethyl-6,7-dimethyl-lumazine has been converted into a deuteriated flavin in a reaction which simulates the biosynthesis of riboflavin.

6,7-DIMETHYL-8-D-RIBITYL-LUMAZINE (1), the precursor of riboflavin (5) in certain micro-organisms,[1] can be converted into riboflavin in a chemical reaction[2] simulating the biosynthetic process. Using deuterium-labelled lumazines, we have studied the chemical reaction and suggest a new mechanism for the formation of flavins by this method.

The n.m.r. spectrum of 6,7-dimethyl-8-D-ribityl-lumazine (1) in D_2O at pH 7 shows that the 7-methyl protons exchange with the solvent, whereas the 6-methyl protons do not (Table 1). Other 8-substituted lumazines (2) and (3), including the 7-monomethyl derivative (4), behave similarly at pH 7—13, thus confirming that the 7-methyl protons exchange. The exchange phenomenon is peculiar to lumazine derivatives with a 'quinonoid' system of double bonds and is not shown by simpler pteridines [*e.g.* (9)].







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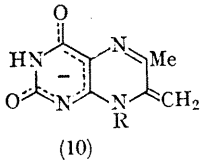
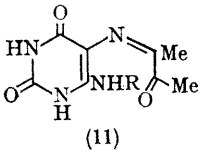


(10)

(1) R^1=D-ribityl, R^2=R^3=Me
(2) R^1=CH_2·CH_2OH, R^2=R^3=Me
(3) R^1=R^2=R^3=Me
(4) R^1=CH_2·CH_2OH, R^2=H, R^3=Me
(5) R=D-ribityl
(6) R=CH_2·CH_2OH
(7) R=Me
(8) R=D-ribityl-5′-phosphate



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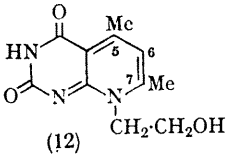


(12)

TABLE 1

^1H *n.m.r. spectra determined at* 60 *MHz.* (τ *values*)

Compound	6-Me	7-Me	Solvent
(1)	7·35	7·12	D_2O (pH ~7)
	7·35	—	D_2O (pH ~7)[a]
(2)	7·06	6·85	CF_3·CO_2H
	7·93	—	NaOD (pH 13)
(3)	7·02	6·88[b]	CF_3·CO_2H
(4)	—	6·83	CF_3·CO_2H
	—	—	NaOD (pH 13)
(9)	7·55	7·53	NaOD (pH 13)
(12)	7·00	6·91	CF_3·CO_2H
	—	—	NaOD (pH 13)

[a] After 15 min. at 100°.
[b] Height of peak decreased by about 50% after heating in D_2O for 1 hr.

We attribute the relative acidity of the 7-methyl protons to the intermediate formation of a highly delocalized anionic species (10), rather than to successive hydration[3] and ring-opening of the lumazine derivative followed by enolization of the resulting methyl ketone (11), because:

(a) Riboflavin-5′-phosphate (8) shows[4] a similar exchange of the methyl protons at position 7. No ring opening is possible in this case.

(b) Studies of related heterocyclic systems show similar exchange phenomena. Thus the protons in *both* methyl groups of the 8-substituted 5,7-dimethylpyrido[2,3-*d*]-pyrimidine (12) undergo exchange in D_2O at pH 13. Were ring-opening to take place in this case it would involve complete fission of the ring-system to a pyrimidine and acetylacetone under conditions where these fragments do not recombine to give the pyrido[2,3-*d*]pyrimidine (12).

(c) The mono-oxime of biacetyl, a simple analogue of the methyl ketone (11), undergoes exchange of the methyl ketone protons only very slowly when heated in D_2O at 100°.

When the labelled lumazine (13) was heated under reflux in D_2O at pH ~7 (phosphate buffer) it was converted (*cf.* ref. 2) into the corresponding flavin (14), the n.m.r. spectrum of which showed that the aromatic 8-proton and the 6-methyl protons had been replaced by deuteruim (see Table 2).

Assignment of the n.m.r. signals in the deuteriated flavin (14) was based on (a) the report by Bullock and Jardetzky[4] that the high-field signal in the aromatic region is due to the 8-proton, and the high-field signal in the methyl region is due to the 6-methyl protons and (b) on our comparison (Table 2) of the spectrum of lumiflavin (3) with that of its 6-ethyl analogue.

Clearly, one of the lumazine molecules must donate a C_4 unit *in some specific way* to the second lumazine molecule to form the *ortho*-xylene ring of the flavin. We suggest the mechanism illustrated in the Scheme for the *in vitro* reaction.

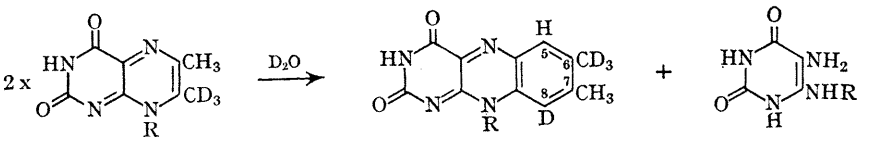
The initial step (a) involves nucleophilic attack by the potential carbanion (15) resulting from one of the lumazine molecules (the acceptor) at position 6 of a hydrated pteridine (16) formed from the second lumazine molecule (the donor) with the formation of an intermediate adduct (17) (*cf.* addition reactions of 7,8-dihydropteridines[5]). It seems that

TABLE 2

^{1}H *n.m.r. spectra determined*[a] *at* 100 *MHz.* (*τ-values*)

Compound	H-5	H-8	6-Me	7-Me	6-CH_2·CH_3
(6)	1·66	1·77	7·32	7·20	
(14)	1·66			7·21	
(7)	1·65	1·85	7·30	7·16	
6-Ethyl-7,9-dimethylisoalloxazine	1·66	1·88		7·17	6·98 (q) 8·52 (t)

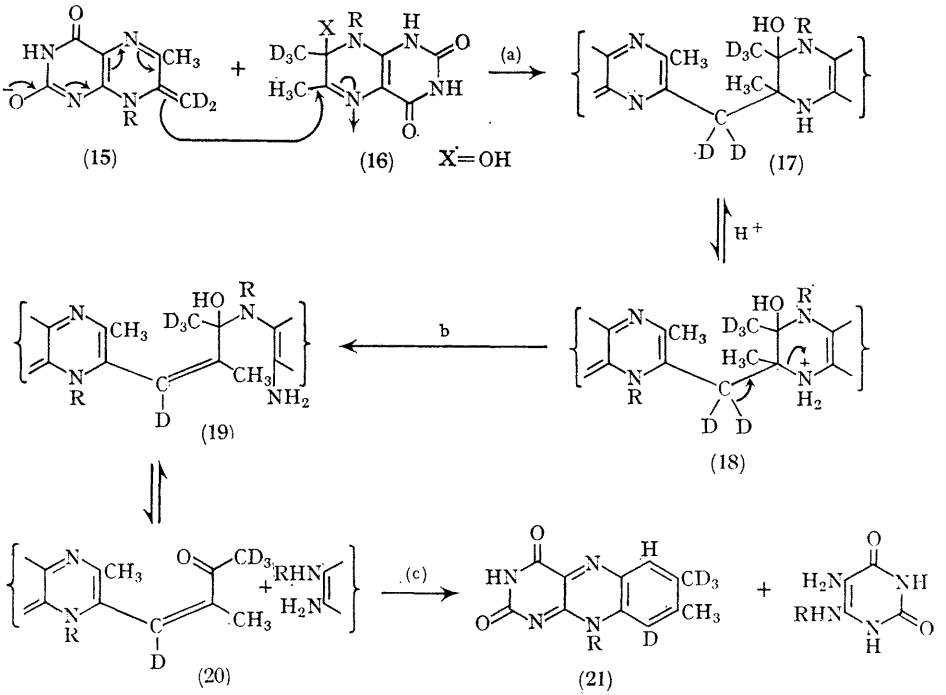
[a] Spectra determined for solutions in CF_3·CO_2H: all signals were singlets unless otherwise stated.



(13) (14)

the second lumazine molecule must react as a 7,8-dihydropteridine in order to explain nucleophilic attack at position 6. Were this lumazine molecule to react in the quinonoid form (1—3), nucleophilic attack would undoubtedly[3] occur at position 7, and the resulting flavin would not be labelled as described above.

While the exact sequence of events may not be that outlined, we believe our results to be significant as affording a possible explanation for the fact[6] that in the biochemical system there are two binding sites on the enzyme surface; one binding a lumazine molecule in such a way that it functions as donor of the C_4 moiety [*e.g.* (16, X = S-enzyme)],



SCHEME. For clarity, deuterium labels are shown only on carbon

We suggest that subsequent steps involve (b) ring-opening of the protonated form (18) of the initial adduct to give the simple carbinolamine (19) which will be in equilibrium with the ketone (20) and a diaminouracil fragment. Cyclisation (c) of the ketone (20) will give the flavin (21) with the correct distribution of deuterium label.

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